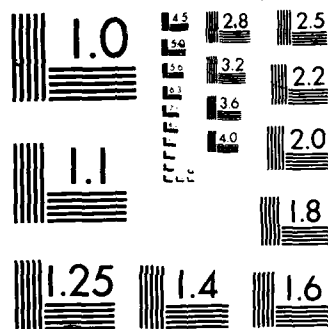


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RED CELL STORAGE STUDIES

Annual Report

STEPHEN B. SHOHET, M.D.

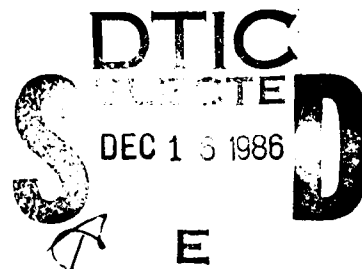
JUNE 4, 1984

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San Francisco, California 94143



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<p>Since the initiation of this contract, we have been involved in two general areas concerning red cell storage. In the first general area, we have been correlating in vitro measurements of red cell deformability as measured in the ektacytometer with in vivo survival of red cells and radioactive chromium-tagged red cells. In the second area, we performed characterization of the senescent cell antigen which is likely to be produced during cell storage. In these studies, we have preliminary evidence that this senescent cell antigen is antigenically-related to band 3 and that it may represent a proteolysis product of the external surface portion of this membrane protein.</p>					
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U.S. ARMY GRANT
RED CELL STORAGE STUDIES
ANNUAL REPORT - YEAR 01

Since the initiation of this contract, we have been involved in two general areas related primarily to objective numbers 1 and 3, as described in the original proposal.

In the first general area, we have been correlating in vitro measurements of red cell deformability as measured in the ektacytometer with in vivo survival of red cells, as measured by our collaborator, Dr. Ernest Beutler, with radioactive chromium-tagged red cells. In general, the ektacytometric measurements have given us some insight into the influence of changes in red cell surface area, membrane stiffness and whole cell viscosity which might have occurred during the storage process. Up to now, we have studied blood from 12 normal subjects which has been stored for 42 and 29 days CPDA-2. As can be seen from Table I, a usefully-wide spread of survival was obtained when these storage cells were subsequently relabeled and then reinfused into the autologous donors. Interestingly, the correlation with the ektacytometric measurements of cell deformability and the survival times, in vitro, has been good and there has been an especially good correlation at the extremes of short and long survival, as shown by comparing the enclosed ektacytometric tracings of donors JW and EB with good 24 hour viability and with the tracings of patients GR, RA and MR with extraordinarily limited 24 hour viability. It is, of course, important, to note that similar correlations with the glucose consumption and ATP depletion were also seen and the present experiments do not allow us to distinguish between the three measurements of ektacytometric deformability, final glucose levels, and final ATP levels as the most specific predictor of in vivo survival.

To attempt to distinguish amongst these three possibilities, further studies will now be conducted utilizing a fixed time of in vitro storage, but varying the hematocrit of the cells being stored. This should modify glucose consumption and ATP depletion rates, appreciably, and may have less effect on cell deformability changes. In addition, to validate that the cell deformability changes are primarily due to cell surface area loss, we will perform lipid analyses of the stored cells and also of ultracentrifuged aliquots of the stored cell media to assay micro vesicular membrane material shed during the storage process. Finally, aliquots of stored cells will be held by our collaborator, Dr. Marguerite Kay, to perform senescent cell antigen analyses, both in the beginning of storage and at the end of storage, (when the survival studies will be done). These studies should give us some insight as to the mechanism of the deformability loss which we have observed here.

The second area of studies that we have performed during the past year includes further efforts to characterize the senescent cell antigen which is likely to be produced during cell storage. In these studies, done in collaboration with our sub-contractor, Dr. Kay, we now have preliminary evidence that this senescent cell antigen is antigenically-related to band 3, and that it may represent a proteolysis product of

the external surface portion of this membrane protein. In brief, the senescent cell antigen was mapped along the band 3 molecule using topographically-defined fragments of band 3. Binding of IgG eluted from senescent red cells to defined proteolytic fragments of band 3 in immunoblots was performed in addition to 2-dimensional peptide mapping of senescent cell antigen, and band 3, and some of its proteolytic fragments. It was found that there was a high degree of homology of the senescent cell antigen and a portion of band 3. This portion was subsequently mapped to a region on the extracellular (outer surface) segment of band 3 which includes approximately 1/3 of the anion transport region of this molecule. Taken together, the data suggests that the critical antigenic determinants of the senescent antigen that are recognized by physiologic IgG autoantibodies are on an external portion of a transmembrane fragment of band 3 that has lost approximately 40,000 daltons of its cytoplasmic (N-terminal) segment which includes part of its anion transport region. Hence, it appears that some age-specific proteolytic cleavage of band 3 occurs during in vitro cell aging to produce a new antigenic site which is recognized as the senescent cell antigen.

These findings are presented in further detail in Appendix B of this report in the form of a manuscript which has been just submitted for publication in the Proceedings of the National Academy of Sciences. Encouraged by these findings, and as noted above, we will now perform determinations of the amount of senescent cell antigen which appears in the in vitro storage samples used for the survival and ektacytometric studies discussed above.

Finally, in terms of studies proposed for the coming year, we would like to note the following: A crucial assumption in many of the preservation studies, as well as in other studies involving sequential changes in the red cell membrane during both in vivo and in vitro maturation, is that cell density is proportionate to the duration of cell aging. In anticipation of the importance of this assumption for the storage studies which we are conducting, we now feel that an in vivo iron or glycine cohort labeling and survival study, coupled with density fractionation is necessary to unequivocally test this assumption. We anticipate that next year, with the collaboration of Dr. Shobana and Dr. Beutler, we may be able to perform a few control studies of this nature utilizing Stratan gradient density analyses of the labeled cells.

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TABLE I: Red Cell Survival (24 h chromium viability) in Relationship to Length of Storage and Metabolic Parameters.

Red Cells are stored for various periods of time in CPDA-2 and the samples are ranked according to percent viability. The viability and metabolic parameters shown here should be compared with the ektacytometric tracings which are included in Appendix A and which are also arranged in decreasing order of deformability. Note that the maximum height of the deformability curves, the Deformability Index, (DI) falls progressively as survival falls. Also note that reincubation prior to analysis in BSKG (a treatment to decrease accumulated lactate) does not influence the DI appreciably, but only shifts the curve symmetrically to the left, suggesting a reversible cell size change. Similarly, incubation with Inosine and Pyruvate to restore 2,3 DPG and, perhaps, ATP levels, also does not appreciably affect the DI level, but results in a further shift of the curve to the left. Both of these observations suggest that irreversible membrane lipid loss accounts for the deformability change which we have seen to be correlated with survival.

TABLE I

DONOR	LENGTH OF STORAGE (DAYS)	HEMATOCRIT (%)	ATP (μ MOLES/gm Hb)		2,3 DPG (μ MOLES/gm Hb)		GLUCOSE (mg%)		HEMOLYSIS (%)		RBC MASS (ml RBC)		24 Hr. VIABILITY (%)	
			INITIAL	FINAL	INITIAL	FINAL	INITIAL	FINAL	51CR	TRUE	51CR	TRUE	51CR	TRUE
JW	7	77.8	3.34	4.15	14.93	12.18	286.9	0.05	1114.4	1124.1	100.0	100.9		
EB	7	82.0	4.44	5.02	16.17	18.36	265.5	0.07	1460.6	1507.9	97.8	100.9		
PS	35	81.3	4.42	2.09	12.28	0.21	1.3	0.43	1677.7	1664.1	76.7	76.1		
PH	35	79.2	4.10	2.44	11.79	0.11	28.2	0.76	1894.7	1828.4	70.1	67.7		
SF	42	76.8	4.17	1.29	13.01	0.21	15.8	1.46	1742.4	1670.2	64.9	62.2		
RB	35	78.7	3.91	1.62	10.99	0.21	46.6	1.56	1344.3	1248.4	73.2	68.0		
JP	42	74.0	4.45	1.91	11.55	0.16	85.1	1.03	2504.9	2421.0	72.3	69.9		
CM	35	81.8	3.69	1.96	13.02	0.25	6.6	1.10	1575.6	1535.5	71.3	69.5		
IE	42	79.7	5.17	1.79	14.62	0.23	55.6	1.10	1623.5	1469.9	68.0	61.6		
MR	49	81.9	4.43	0.34	12.57	0.22	0	1.96	2798.2	2422.9	16.3	14.1		
RA	49	79.1	3.93	0.38	12.99	0.26	0	2.81	3193.2	2736.9	9.6	8.2		
GR	42	79.2	4.01	0.60	13.72	0.13	0	1.21	2263.2	1777.9	16.9	13.3		

APPENDIX A

Ektacytometric tracings showing red cell deformability under constant shear and varying osmotic conditions.

The tracings are arranged in order of decreasing, overall, deformability. Please compare to the order of in vivo red cell survivals in Table I.

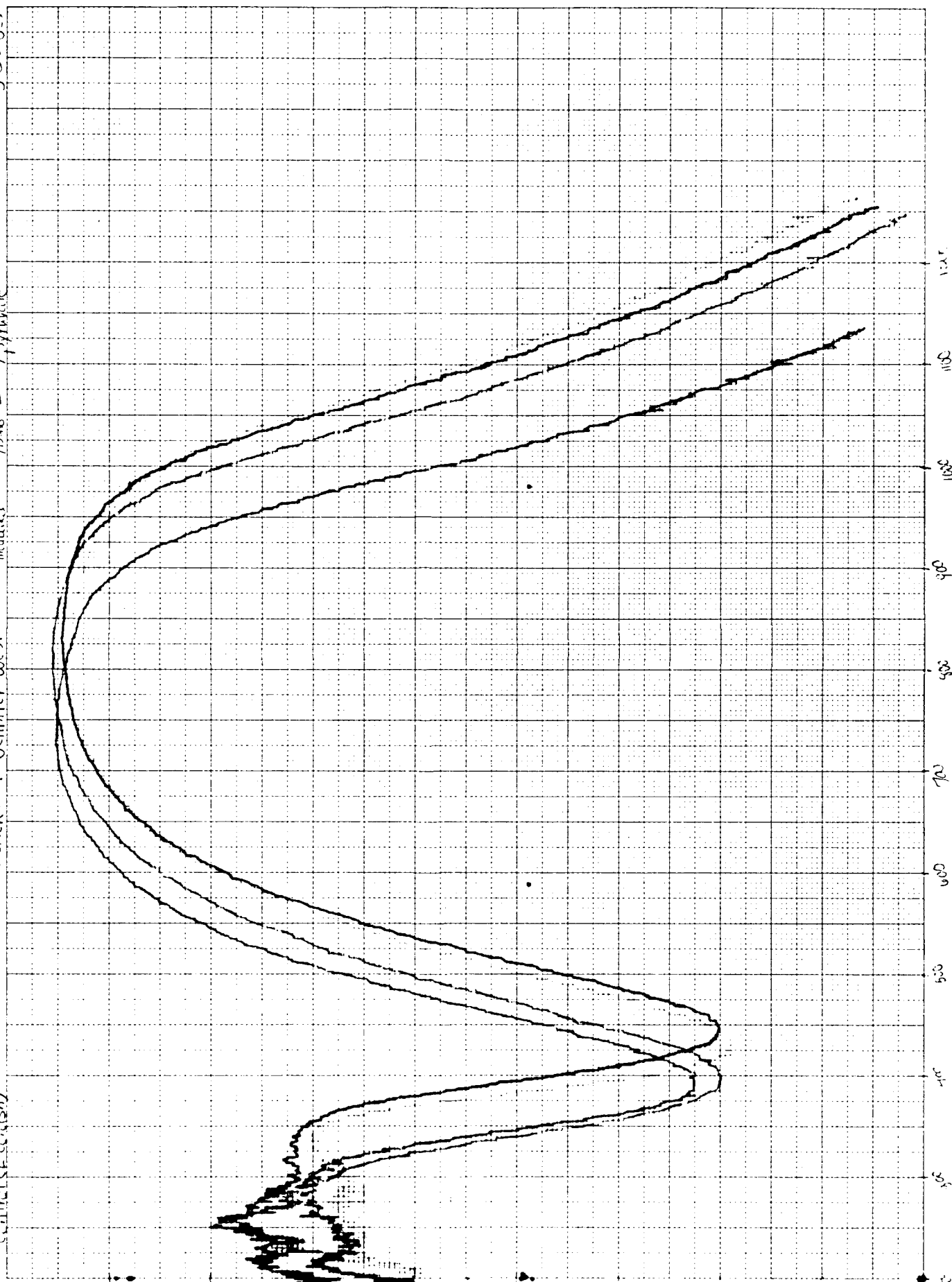
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Donor #9 Jennifer West

mobility - w/BKG - ~~in~~ ^{incubate} by pyruvate

Cute 78%

5-03-83

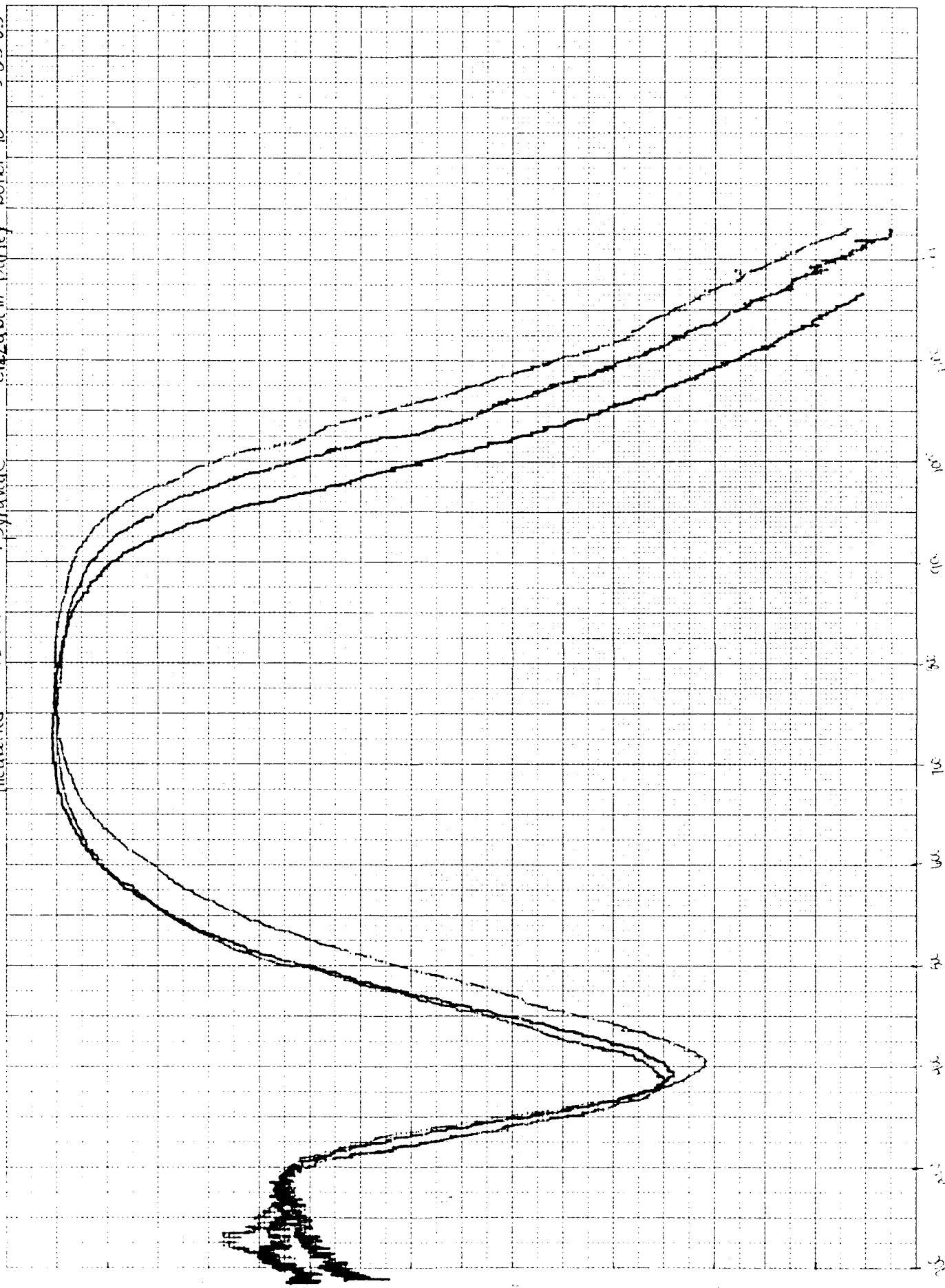


U

note 34%

Elizabeth Burley Donor #10 5-35-83

not incubated - w/BSKG - w/insane
pyruvate



4

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19 D120
Paul Hyslop

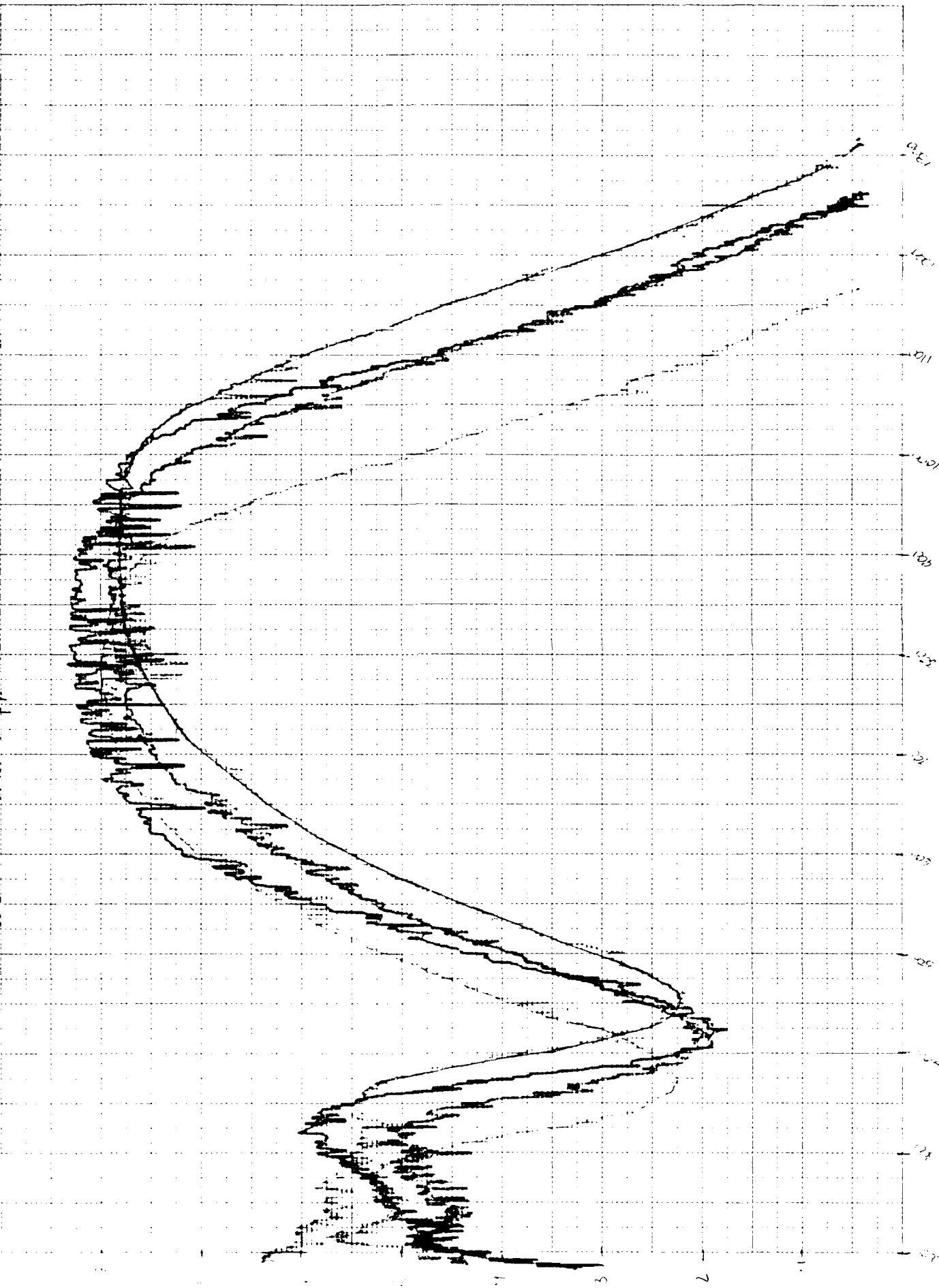
7.70.200
unlabeled
4B505

79D120
unlabeled
4B505

DONOR #2

8.2 D120
Control (Jill)

3.2-8.3



5

)

)

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0.0 0. 7.4

— Shinya Jafu
(Daker 1)

0.76

... incubated
the w/ B's

0.79

— incubated
pyruvate, inosine

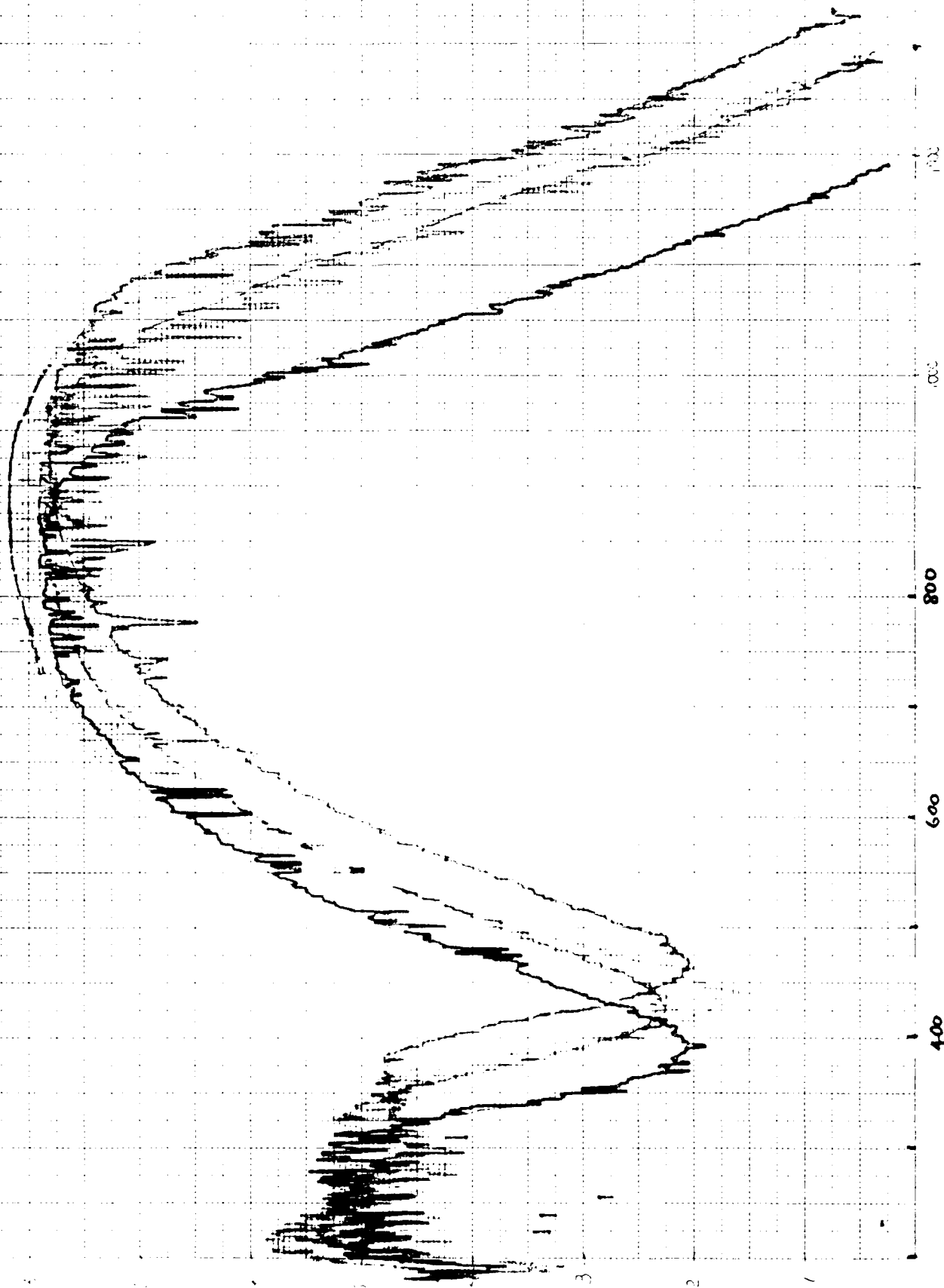
0.81

Control
(Hahn)

)

)

Reactions
Samples
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RENDER 44 (R 44.4)

)

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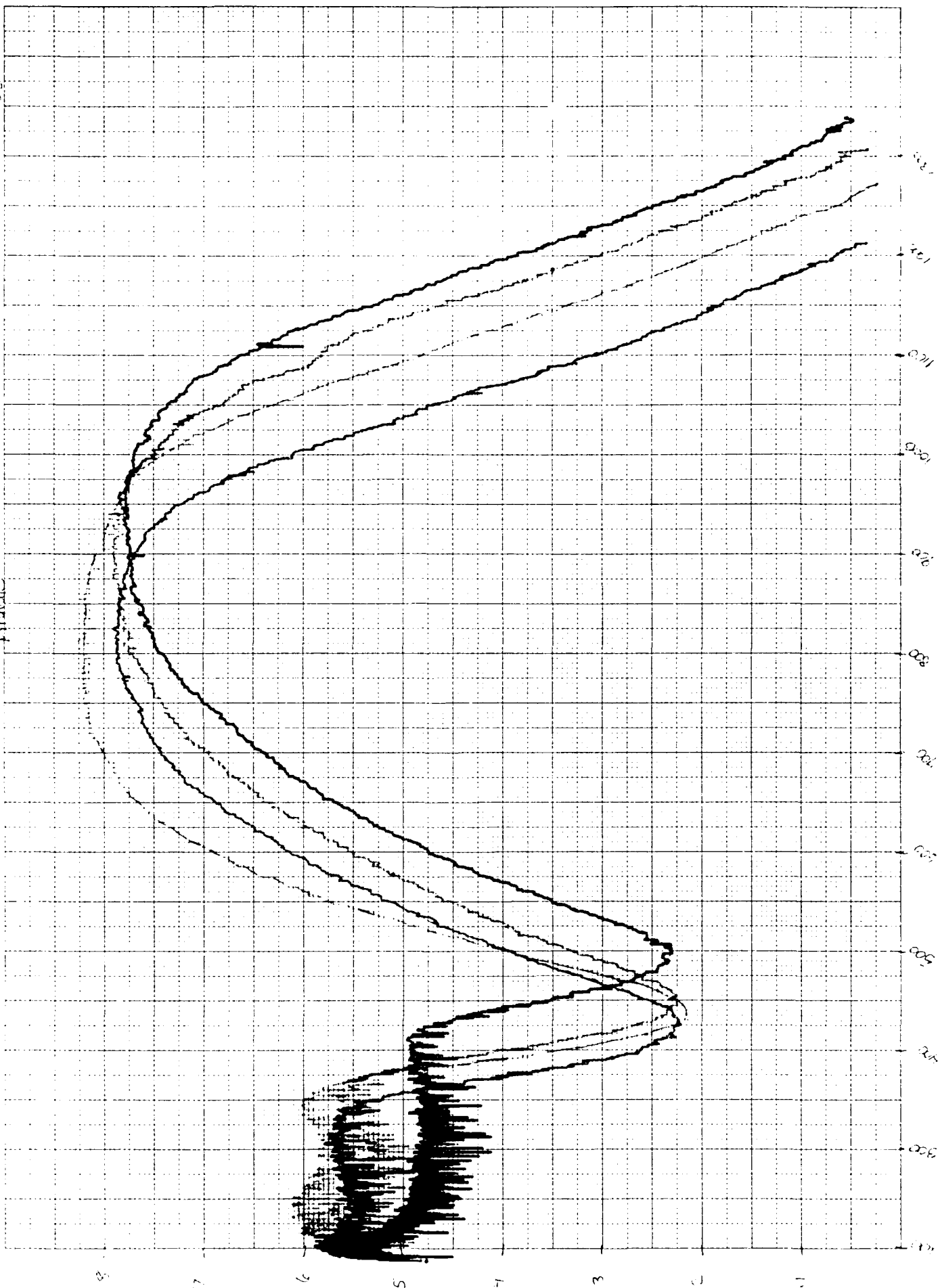
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HEWLETT PACKARD 9270 1003

— RENDER — w/ 100%
— w/ 100%
— w/ 100%

Control (Sketch)

7983



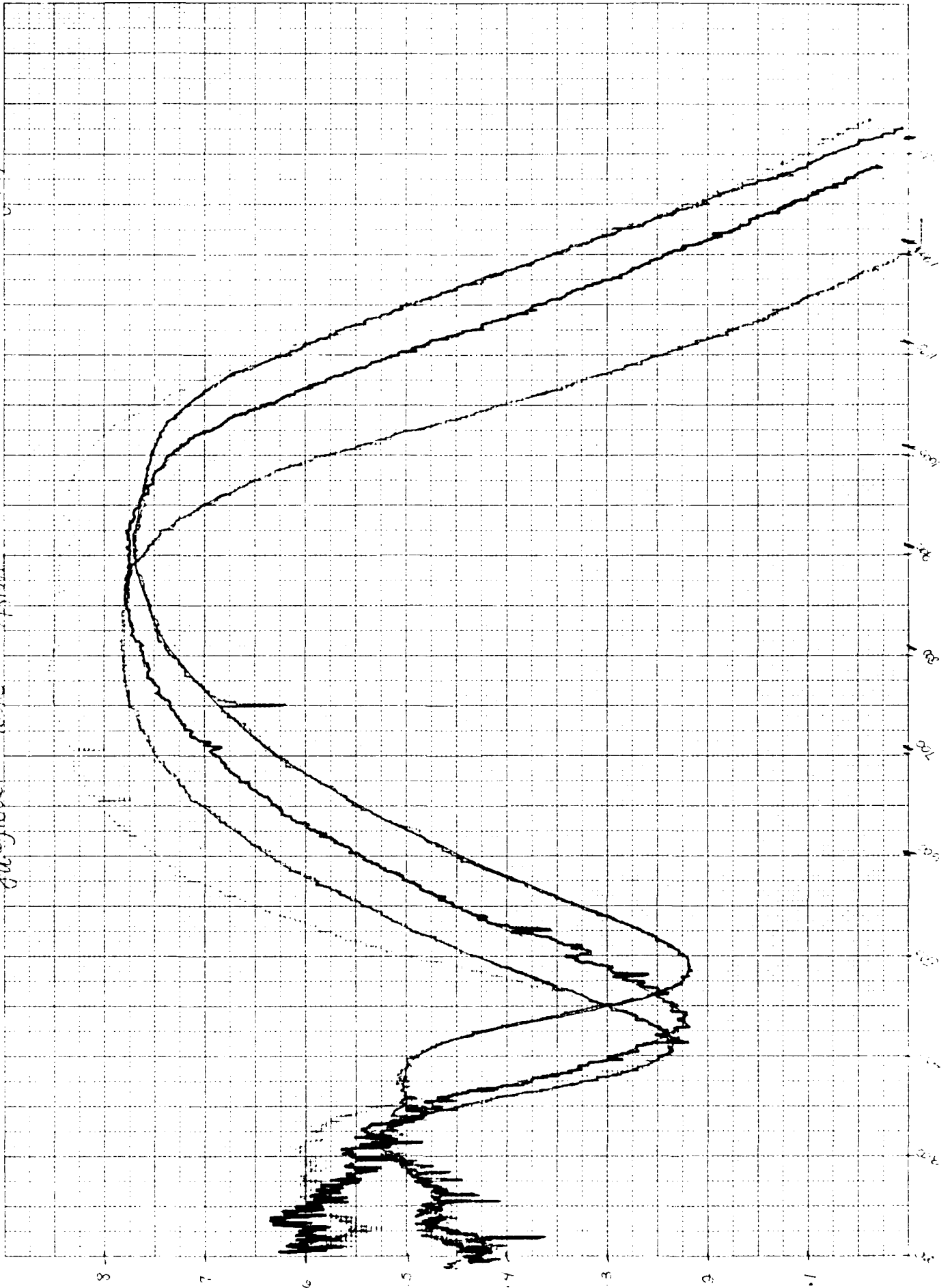
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Device #7

Jeffrey Porter - w/BSK - w/Power

Control (Mingot)

3-21-83



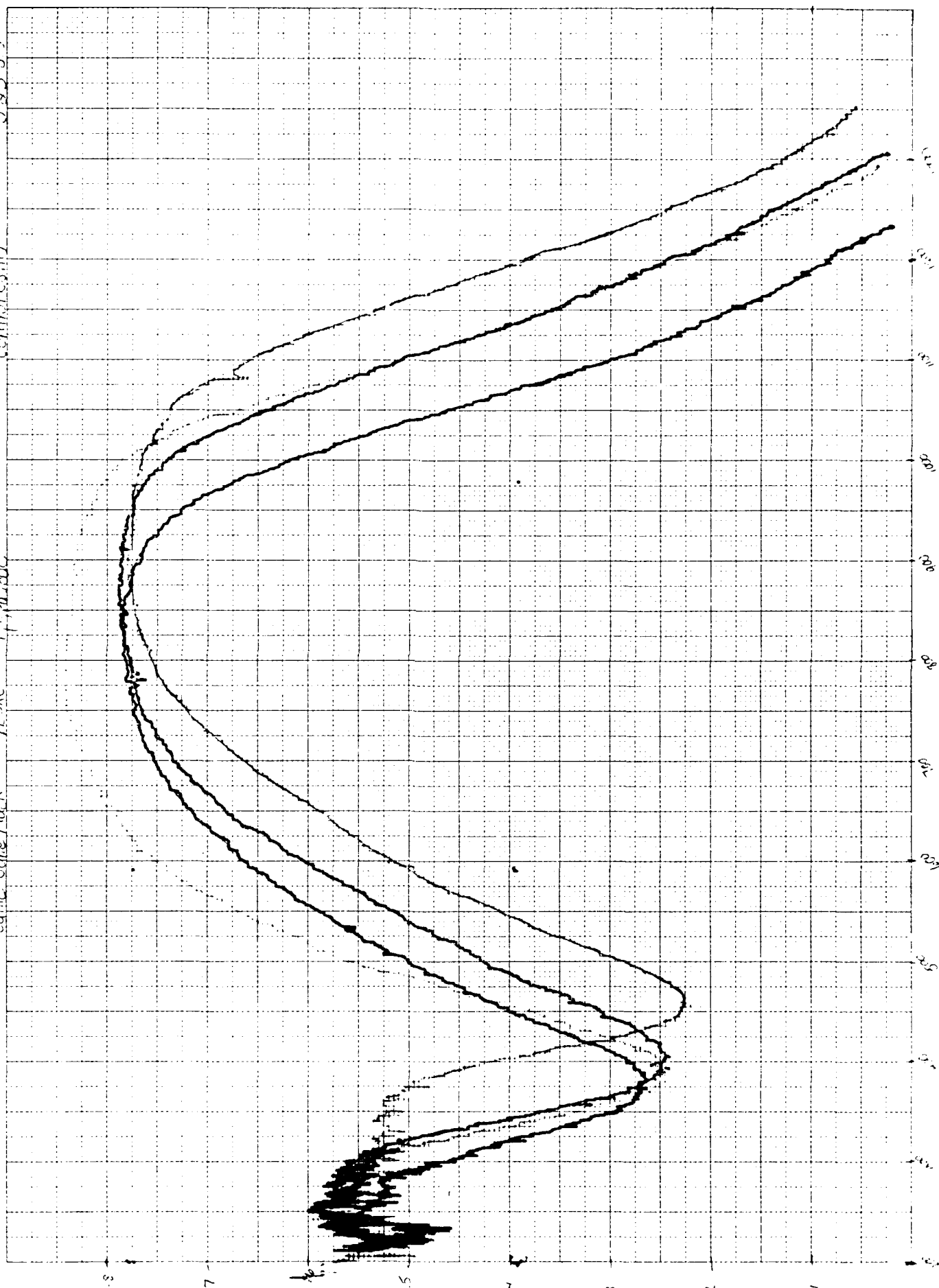
Unit 81%

Donor #8

--- Colloidal Japane Mark --- w/BK --- w/BK --- w/BK

--- Control (5.11)

3.23-83

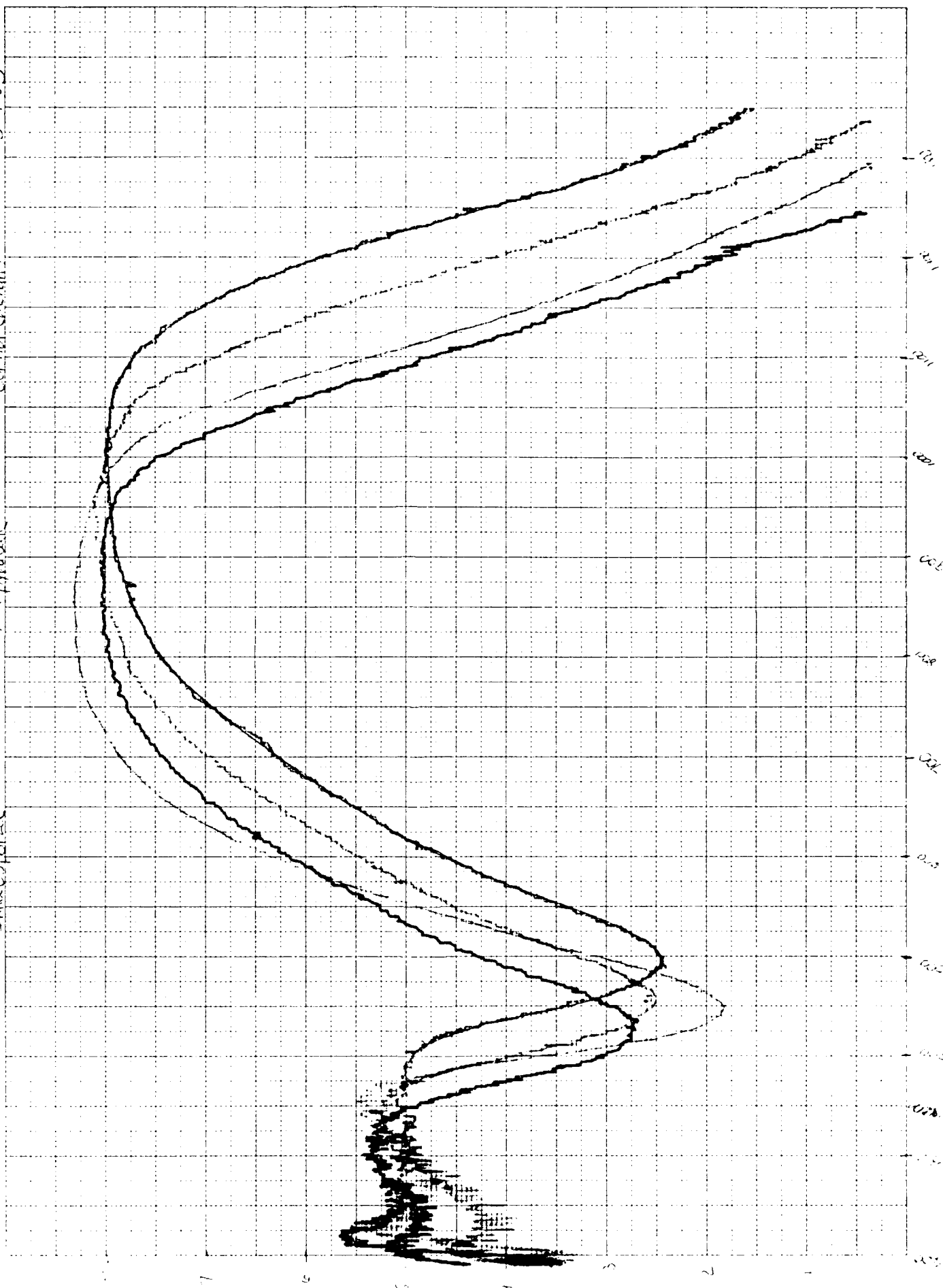


Donor #3

— Intra Esophageal — w/ Pacer — w/ 100% sine wave

--- Control (Atrium)

3-7-83



HILWETT PACKARD 9270-1023

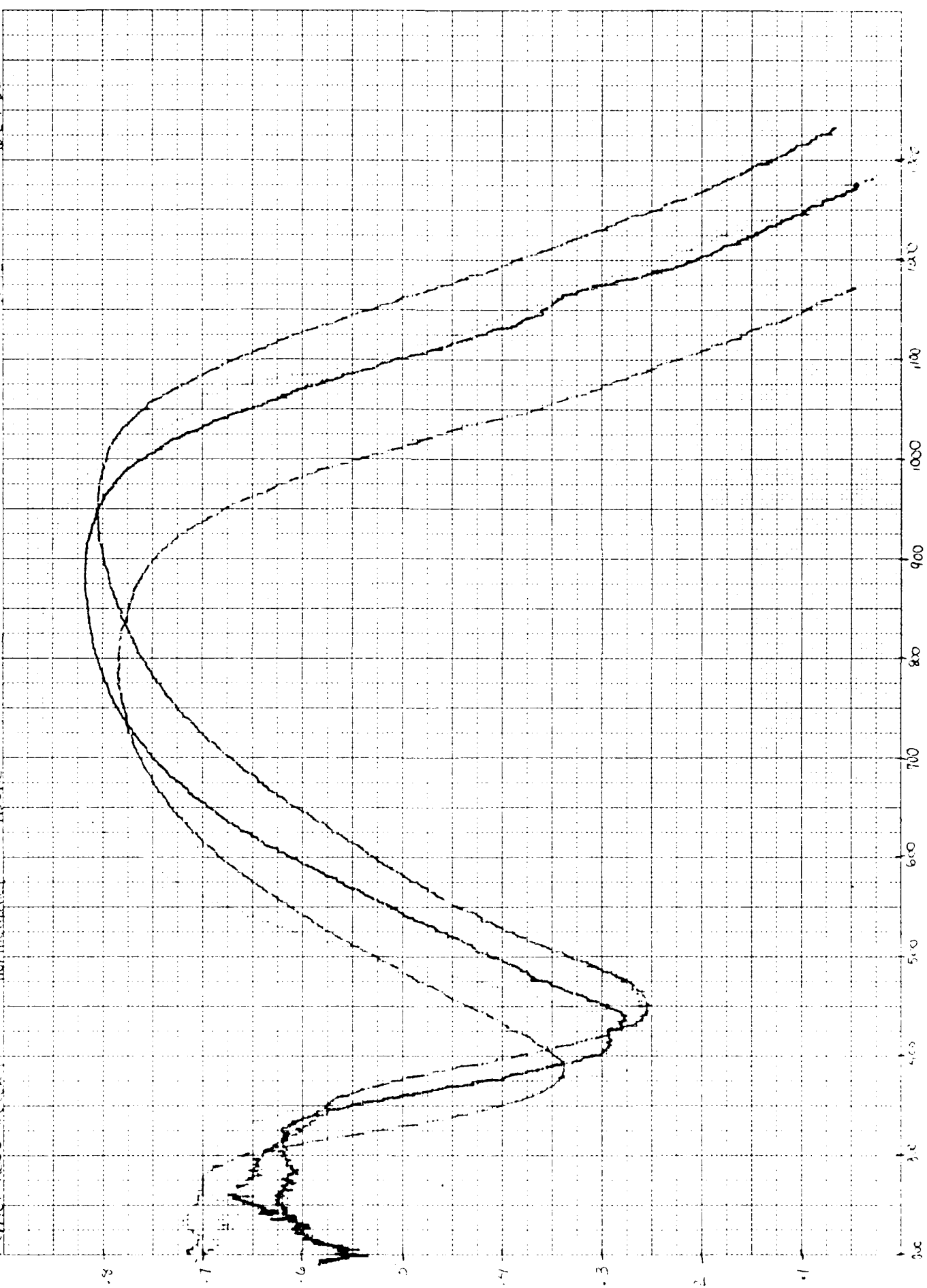
CRITE 510%

Metric K Res. Dev. 17

- not incubated - 1735KG

- 1735KG

6-24-83

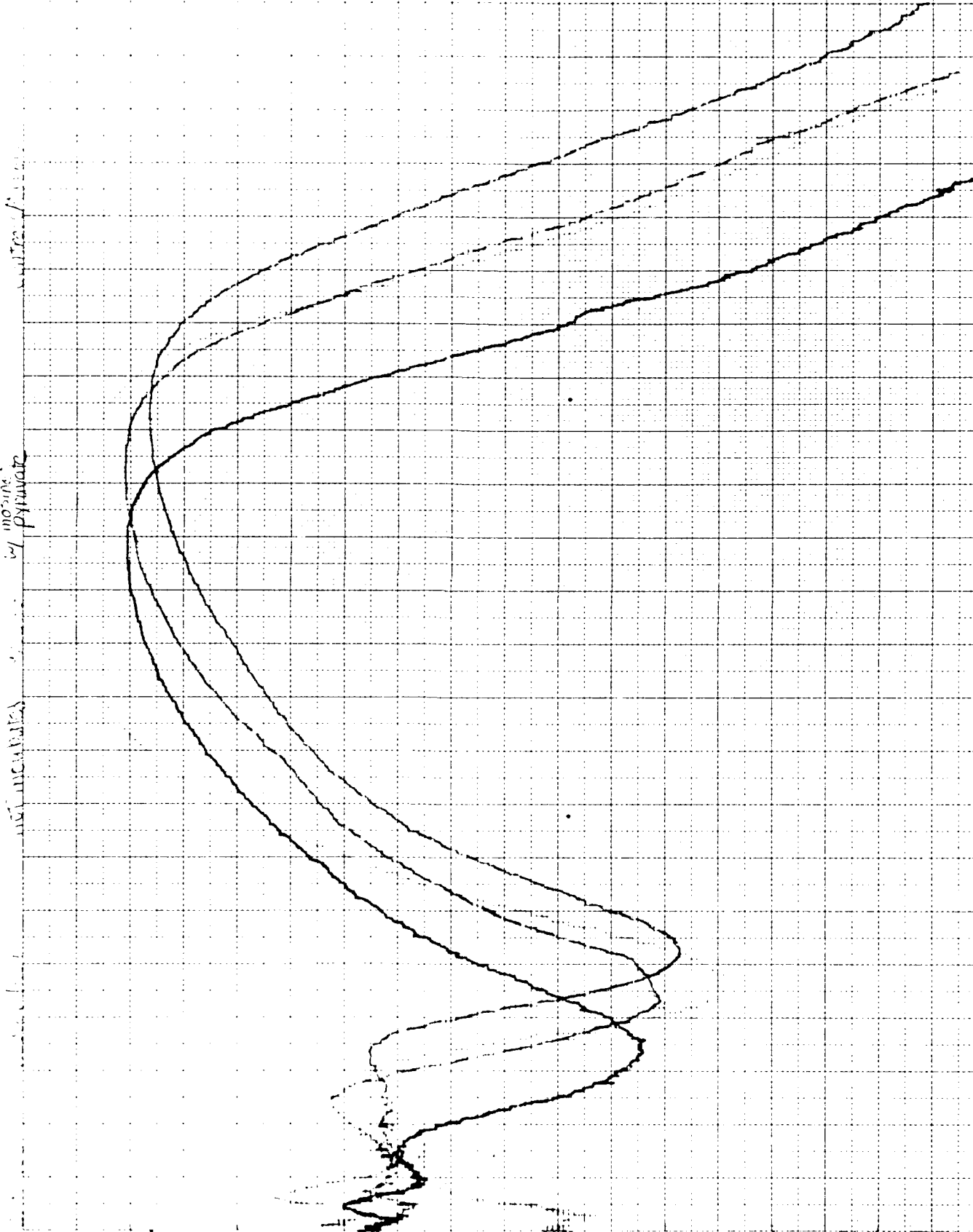


W/ 100%
pyruvate

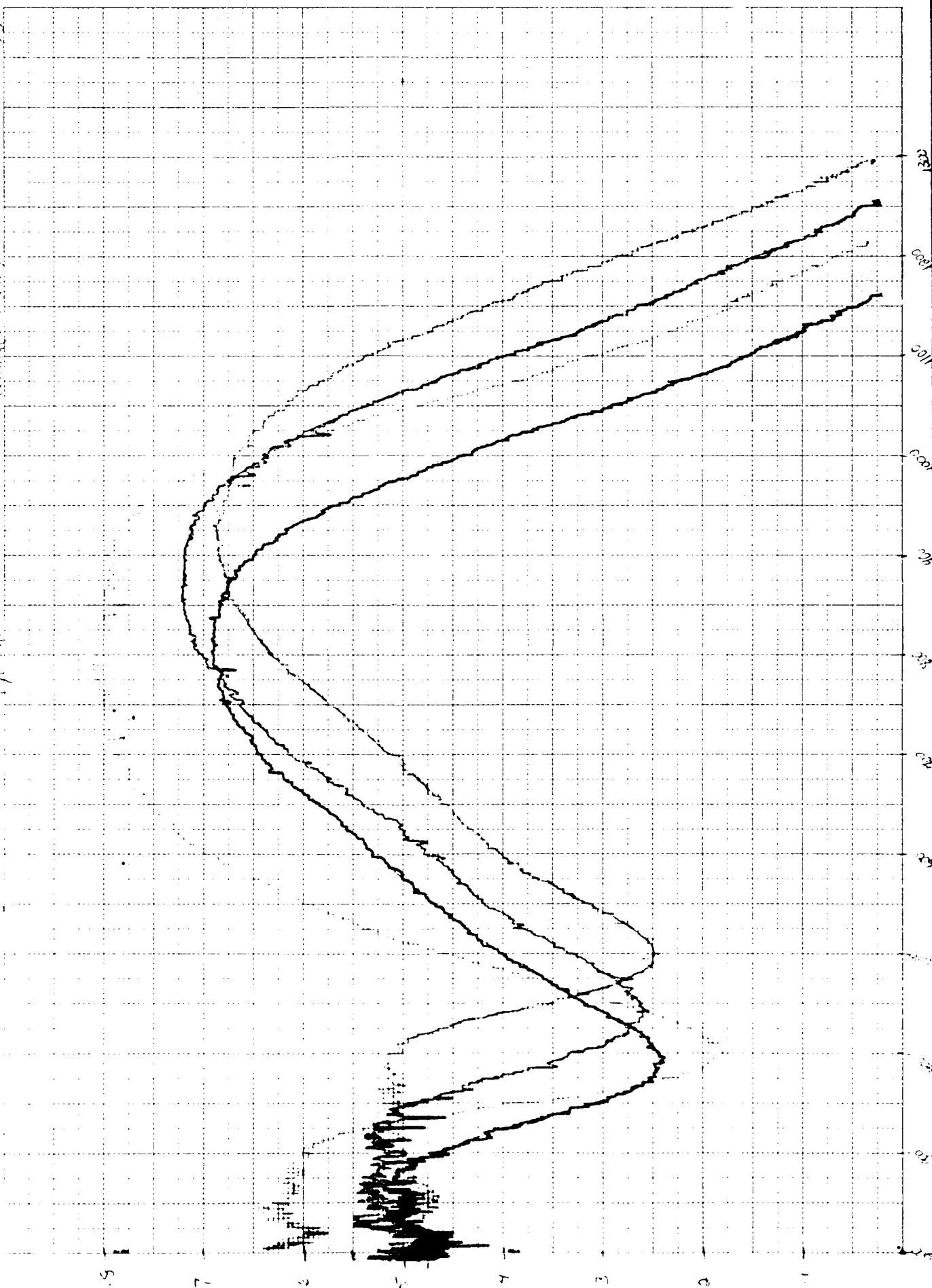
W/ 100%
pyruvate

W/ 100%
pyruvate

W/ 100%
pyruvate



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— (Control) — (1000 Hz) — (1000 Hz) — (1000 Hz)



Classification: Cell Biology

Title: Localization of senescent cell antigen on band 3

Key Terms: (fragments of band 3/two-dimensional peptide mapping/aging antigenic determinants/anion transport region/immunoblotting)

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Abbreviations: RBC, red blood cells; PBS, phosphate buffered saline; PMSF, phenylmethanesulfonyl fluoride, PAS, periodic acid Schiff's; DFP, diisopropylfluorophosphate.

ABSTRACT

Senescent cell antigen is a glycosylated 4.5 region polypeptide that appears on the surface of senescent and damaged cells. Appearance of the senescent cell antigen initiates specific binding of immunoglobulin (Ig) G autoantibodies to it and the removal of red cells. Previous experiments suggested that the senescent cell antigen may be immunologically related to band 3.

In the present studies, senescent cell antigen was mapped along the band 3 molecule using topographically defined fragments of band 3. Both binding of IgG eluted from senescent red blood cells ("senescent cell IgG") to defined proteolytic fragments of band 3 in immunoblots, and two-dimensional peptide mapping of senescent cell antigen, band 3, and defined proteolytic fragments of band 3 were used to localize senescent cell antigen along the band 3 molecule. Senescent cell antigen was mapped to a region on the extracellular (outer-surface) portion of band 3 that includes ~30% of the ~17,000 Da anion transport region. The data suggest that the critical antigenic determinants of the senescent cell antigen that are recognized by physiologic IgG autoantibodies reside on an external portion of a naturally occurring transmembrane fragment of band 3 that has lost an ~40,000 Da cytoplasmic (N-terminal) segment and part of

the anion transport region. A critical cell age specific proteolytic cleavage of band 3 appears to occur in the transmembrane, anion transport region of band 3.

INTRODUCTION

The senescent cell antigen is a glycosylated polypeptide that appears on the surface of senescent and damaged red cells (1-14). It is recognized by the antigen binding, Fab, region (3,4) of a specific immunoglobulin (Ig) G autoantibody in serum which attaches to it and initiates the removal of cells by macrophages (5). Although the senescent cell antigen was first demonstrated on the surface of senescent human erythrocytes (1,2), it has since been demonstrated on the surface of lymphocytes, polymorphonuclear leukocytes, platelets, embryonic kidney cells, and adult liver cells (4).

It was postulated that the senescent cell antigen was a component of the band 4.5 region that was derived from band 3 (6) based on extraction and isolation conditions, relative molecular weight, and its characterization as a glycosylated peptide (4). Experiments designed to test this hypothesis revealed that the senescent cell antigen is immunologically related to band 3 (9-11). Both band 3 and senescent cell antigen abolished the phagocytosis-inducing ability of IgG eluted from senescent cells which binds specifically to the senescent cell antigen.

Spectrin, bands 2.1, 4.1, actin, glycophorin A, PAS staining bands 1-4, and desialylated glycophorin A and PAS staining bands 1-4 did not alter the phagocytosis-inducing ability of senescent cell IgG. In addition, monospecific rabbit antibodies to both purified band 3 and the senescent cell antigen reacted with band 3 and its proteolytic products M_r ~60,000, 40,000 and 18-26,000 as determined by immunautoradiography of RBC membranes indicating that these molecules share common antigenic determinants. These lower molecular weight band 3 related polypeptides are thought to represent proteolytic fragments generated in vivo because they are observed in membranes from old cells prepared with the protease inhibitors diisopropylfluorophosphate, EDTA, EGTA, and phenylmethylsulfonyl fluoride, but not in membranes from young cells (11).

In the present study, binding of IgG eluted from senescent red cells ("senescent cell IgG") to proteolytic fragments of band 3 presumably generated in vivo and those produced in vitro by protease treatment are examined in order to locate the position of the senescent cell antigenic determinants on the band 3 molecule. The fragments are referred to by the nomenclature of Steck, et al. (15). The M_r ~55,000 fragment of band 3 (CH-55) and its complementary M_r ~38,000 fragment (CH-38) were produced by mild α -chymotrypsin treatment of intact red cells (15,16).

The M_r ~41,000 cytoplasmic fragment (TR-41) of band 3 and associated fragments were produced by treatment of inverted vesicles depleted of peripheral membrane proteins by alkali treatment (17). Treatment with α -chymotrypsin at both sides of the membrane yielded the M_r ~17,000 fragment (CH-17; 15, 16, 18). In addition, these fragments of band 3, and senescent cell antigen were analyzed by two-dimensional peptide mapping.

MATERIALS AND METHODS

Cell separation. Red blood cells (RBC) were separated into young, middle-aged, and old populations on Percoll (Pharmacia) gradients as previously described (5). Four mls of blood were mixed with 20 ml of Percoll diluted 1:10 with 10 X Dulbecco's phosphate buffered saline (PBS). Gradients were centrifuged at $18,450 \times g_{av}$ ($25,950 \times g_{max}$) for 30 min. at 4°C . Platelets, white cells, and reticulocytes formed bands at the top and were removed. Young RBC were in the least dense fraction (ρ 1.090), middle-aged cells in fractions 1.10-1.11, and old cells in the most dense fraction (ρ 1.120) as determined by ^{59}Fe labeling in situ (5). Old cells used for the studies described here represented 0.6% of the total cells. Middle-aged cells from the denser middle-aged fractions were used. Red cells were washed three times in 20 vols. of PBS by centrifugation at $3000 \times g$ for 10 min., lysed, and the membranes washed with 5mM sodium

phosphate buffer, pH 7.4, containing 1 mM EDTA, 1 mM EGTA, 5 mM DFP and 100 μ g/ml phenylmethanesulfonyl fluoride as protease inhibitors.

Isolation of IgG from Senescent RBC and Senescent Cell Antigen. IgG was isolated as previously described (3,4). Briefly, middle-aged and old RBC were washed three times with 50-100 volumes of PBS, pH 7.4. RBC membranes were prepared by digitonin lysis and washed three times with PBS. IgG was eluted with 0.1M glycine-HCl buffer, pH 2.3. Eluates were neutralized with 1 N NaOH and concentrated using an Amicon Diaflow with a PM 10 filter. IgG was isolated from eluates by affinity chromatography with protein A conjugated to Sepharose 4B. Senescent cell antigen was isolated by affinity chromatography with senescent cell IgG conjugated to Sepharose 4B as previously described (4,6).

Enzymatic treatment of erythrocytes. Washed erythrocytes were incubated with PBS containing 1mM ATP and 200 μ g/ml Chymotrypsin overnight at 24 °C. Chymotrypsin specifically digests band 3 yielding fragments of ~55,000 and ~38,000 Da (15,16), designated CH-55 and CH-38, respectively (15). The CH-55 fragment appears to have a molecular weight closer to 60,000 in our experiments. Digestion was terminated by the

addition of 5mM DFP. Cells were washed four times with PBS and processed in the same manner as described for intact erythrocytes.

The ~41,000 Da (TR-41) cytoplasmic segment of band 3 was produced by mild α -chymotrypsin or trypsin digestion of spectrin-depleted, NaOH stripped inverted vesicles (17). Digestion was terminated by the addition of 5mM DFP and 200 μ g/ml PMSF. Aliquots were removed for electrophoresis. Vesicles were removed by centrifugation and the cytoplasmic fragments of band 3 were collected by direct addition of DEAE cellulose to the supernate. Fragments were eluted with a salt gradient of 0.2 to 0.5M KCl (17). The complementary fragment of TR-41 remains with the membrane and contains CH-38 and CH-17. This fragment was obtained from the vesicles.

The M_r ~17,000 (CH-17) intramembraneous fragment of band 3 was generated by α -chymotrypsin treatment at both sides of the membrane (15,17,18). Proteolysis was terminated by the addition of 5mM DFP. Membranes were washed twice with 5mM sodium phosphate, pH 8.0 containing 1mM EDTA, 1mM EGTA, and 1mM DFP. The M_r ~19,000 intramembraneous fragment (CH-TR-19) which includes CH 17 was produced by α -chymotrypsin treatment of intact red cells followed by treatment of spectrin depleted inverted vesicles with trypsin (19).

¹²⁵I labeling of intact RBC. Middle-aged red cells were washed five times with 20 vols. of PBS and resuspended in Hank's buffered salt solution. Iodinated p-hydroxyphenylpropionic acid, N-hydroxysuccinimide ester (¹²⁵I Bolton-Hunter reagent, 2.0 mCi; New England Nuclear) was added to 5 cc of the cell suspension, and the cells were incubated at ice temperature for two h. Cells were washed six times with PBS and resuspended to a hematocrit of 50% in autologous serum with citrate-phosphate-dextrose-adenine 1. Cells were stored in autologous serum at 4°C for five days to increase the amount of band 3 breakdown products (9), washed four times with PBS, and membranes prepared as described earlier.

Sodium dodecyl sulfate (NaDodSO₄) polyacrylamide gel electrophoresis. Proteins were analyzed on three different gel systems: 7% NaDodSO₄/polyacrylamide gels, and 6-25% and 12-25% linear NaDodSO₄/polyacrylamide gradient gels using the discontinuous buffer system of Laemmli (20).

Immunostaining of membrane proteins. Immunoautoradiography was performed by the immunoblotting technique of Towbin et al. (21) with the modifications described previously (10), or by the gel overlay method (10). Transfer of polypeptides was monitored by loss of Coomassie blue-staining bands from the gel, and by the appearance of Amido black staining bands on the

nitrocellulose paper. Transfer of polypeptides was >90% efficient. Neither preimmune serum nor protein A bind to red cell proteins under the conditions employed (10).

Two-dimensional peptide mapping. Two-dimensional peptide maps were obtained using the method of Elder et al. (21). Gel slices were excised from 6-25% or 12-25% NaDodSO₄ gels and iodinated in 80 μ l of sodium phosphate buffer with 300 μ Ci Na¹²⁵I (Amersham, Arlington Heights, IL) and 20 μ l of chloramine T for two hr at room temperature. Iodinated slides were washed in 50 cc tubes on a rotator with 13 changes of 10% methanol over five days. They were transferred to perforated stainless steel tissue capsules and dialysed against three to five changes of 3 l of 10% methanol until the radioactivity in solution was at background levels. Lyophilized gel slices were incubated in two changes of 25 μ g of *L*-cymoytrypsin or trypsin in 25 mM ammonium bicarbonate buffer for 20 hr at 37°C. The supernatants were pooled, lyophilized, and dissolved in acetic acid/formic acid/H₂O (15:5:80) so that there were 5 X 10⁵ cpm/ μ l. Samples (0.5 μ l) were applied to cellulose-coated thin layer plates. Electrophoresis was carried out in a Pharmacia electrophoresis chamber at 1 kV and 0°C until the DNP-lysine tracking dye had migrated to 3.5 cm from the edge of the plate. After drying, thin layer chromatography in the second dimension was conducted in 1-

butanol/pyridine/acetic acid/H₂O (32.5:25:5:20). Chromatographs were dried and exposed to Kodak X-Omat film in Dupont cassettes with Cronex lightning plus intensifying screens for one to three days at -80°C.

RESULTS

Band 3 degradation products increase with cell age. Young, middle-aged, and old cells were separated on Percoll gradients. Membranes were prepared and erythrocyte proteins were separated by polyacrylamide gel electrophoresis. Antibodies to band 3 and IgG eluted from senescent cells were used to determine the relative amount of band 3 breakdown products in the membranes of young and old cells by the immunoblotting technique. Antibodies to band 3 bind to band 3 in immunoblots of young, middle-aged, and old red cells (Fig. 1). In addition, antibodies to band 3 bind to two lower molecular weight band 3 polypeptides, M_r ~62,000 and ~40,000, in the membranes of old but not young red cells. Binding to M_r ~62,000 and 40,000 band 3 polypeptides was not demonstrated in membranes from young cells even though the amount of young RBC membranes loaded on polyacrylamide gels was greater than that of old RBC membranes (Fig. 1). Senescent cell IgG, which has a specificity restricted to the senescent cell antigen, bound to band 3 and the M_r ~62,000 degradation product

of band 3 (Fig. 1). The M_r ~62,000 breakdown product of band 3 was detected with senescent cell IgG in membranes of old but not young or middle-aged cells. Senescent cell IgG did not bind to band 3 polypeptide M_r ~40,000 to which antibodies to band 3 bound. Thus, the antigenic determinants recognized by IgG eluted from senescent cells appear to reside on a M_r ~62,000 fragment of band 3.

The naturally occurring M_r ~62,000 band 3 fragment is a transmembrane polypeptide. In order to determine whether the naturally occurring fragment of band 3 spans the membrane and communicates with the extracellular space, intact red cells were labeled with ^{125}I Bolton-Hunter reagent which labels terminal amino and lysine groups. Red cell membrane proteins were separated by NaDodSO₄ polyacrylamide gel electrophoresis before and after alkali treatment. Gels were dried and autoradiographed. Autoradiographs revealed labeling of band 3 and the M_r ~62,000 naturally occurring fragments of band 3 in both untreated and alkali treated membranes (Fig. 2). In contrast, the M_r ~40,000 naturally occurring fragment of band 3 was not labeled. This suggests that the ~62,000 M_r fragment spans the membrane; whereas, the ~40,000 M_r fragment does not. Since the naturally occurring ~62,000 M_r fragment is not removed from membranes by

alkali treatment, the data suggest that it is an integral membrane segment.

Binding of IgG eluted from senescent cells to band 3 and its proteolytic products. As an approach to determining which segment of band 3 carries the antigenic determinants of the senescent cell antigen, binding of senescent cell IgG to band 3 fragments that appear to be generated in vivo in the red cell membrane ("naturally occurring") and those generated by \propto chymotrypsin treatment in vitro was investigated. Red cell membrane proteins from untreated and enzyme treated cells were transferred from NaDodSO₄/polyacrylamide gradient gels to nitrocellulose paper. The paper was overlaid with either antibodies to band 3 or IgG eluted from senescent cells followed by incubation with ¹²⁵I labeled protein A. Immunoautoradiographs obtained by exposing the nitrocellulose paper to X-ray film revealed binding of senescent cell IgG to band 3 and a polypeptide migrating at M_r ~62,000 (+3%; range 60-64,000) in membranes from untreated cells (Fig. 3, lane a). The M_r ~62,000 polypeptide also labeled with monospecific antibodies to band 3. Senescent cell IgG bound to both the M_r ~55,000 transmembrane fragment of band 3 (CH-55) generated by mild \propto chymotrypsin treatment of intact RBC and its complementary M_r ~38,000 fragment (CH-38) (Fig. 3, lane b), and to the M_r ~17,000

transmembrane segment of band 3 (CH-17) (Fig. 3, lane c). However, it did not bind to the M_r ~41,000 cytoplasmic segment of band 3 which is released from inverted vesicles following trypsin or α cnymotrypsin treatment (TR-41) (Fig. 3, lane e), although it did bind to a complementary ~60,000 M_r fragment that remained with the membrane (Fig. 3, lane d). The same results were obtained when the gel overlay rather than immunoblotting technique was used (results not presented).

Peptide mapping analysis of senescent cell antigen, band 3, and defined proteolytic products of band 3. The extent of homology between senescent cell antigen and band 3 and its proteolytic products was evaluated by comparing peptide maps of these polypeptides (Fig 4). Senescent cell antigen shares substantial peptide homology with band 3, the ~60,000 Da fragment remaining with the membrane following removal of the TR-41 cytoplasmic segment, and the CH-38 carboxyl terminal segment of band 3. The peptides present in the peptide map of senescent cell antigen are present in the maps of band 3 and the ~60,000 Da carboxyl segment.

The peptide map of CH-38 does not contain all of the peptides present in the map of senescent cell antigen and it contains additional peptides not found in the map of senescent cell antigen. Approximately 30% of the peptides in the cnymotryptic

map of the CH-17 anion transport segment are found in peptide maps of senescent cell antigen. This suggests that proteolytic cleavage of band 3 may occur within the anion transport region.

Since peptides of both CH-38 and CH-17 appear in the map of senescent cell antigen, an attempt was made to generate a map similar to that of senescent cell antigen by mixing equal amounts of CH-38 and CH-17 on the same map. The resulting map closely resembles that of senescent cell antigen although it contains more peptides than are found in the map of senescent cell antigen. These results suggest that peptides of the senescent cell antigen reside on an extracellular, carboxyl terminal segment of band 3 that includes most of CH-38 and part of CH-17.

Partial overlap between the CH-55 and senescent cell antigen was observed (Fig. 5). This is an expected result since CH-55 contains the CH-17 anion transport region as well as the unrelated TR-41 cytoplasmic portion of band 3.

DISCUSSION

IgG eluted from senescent red cells binds to band 3 and its naturally occurring M_r -62,000 proteolytic product observed in membranes of old red cells prepared with the protease inhibitors DFP, EDTA, and EGTA to avoid artifactual proteolysis. These degradation products of band 3 have not been observed in membranes of young cells (11). The -62,000 M_r proteolytic

product appears to have an intramembranous fragment that is exposed to the extracellular space because it can be labeled from the outside of intact cells with ^{125}I Bolton-Hunter reagent, and remains with the membrane following alkali treatment. The M_r ~40,000 fragment can not be labeled from the outside of cells. Thus, it appears to be a cytoplasmic fragment of band 3 that does not cross the membrane.

IgG eluted from senescent red cells also binds to both the CH-55 and CH-38 transmembrane fragments of band 3 produced in vitro by α -chymotrypsin treatment of intact RBC, and to the CH-17 intramembranous, anion transport segment of band 3. However, it does not bind to the TR-41 cytoplasmic fragment of band 3. Since IgG eluted from senescent cells binds specifically to the senescent cell antigen (2-4), the senescent cell antigen appears to reside on a transmembrane segment of band 3 that lacks the TR-41 cytoplasmic segment. This segment includes at least part of the CH-17 intramembranous fragment containing the anion transport site (18), and segments on the carboxyl terminal side of that site.

Peptide mapping revealed substantial peptide homology between senescent cell antigen and the CH-38 and CH-17 fragments of band 3, and between senescent cell antigen and the ~60,000 Da outer-surface band 3 fragment generated by removal of TR-41.

Peptide homology between senescent cell antigen and TR-41 could not be demonstrated. Thus, results of these studies indicate that both the critical antigenic determinants of the senescent cell antigen and the peptides that comprise the antigen reside on an outer surface segment of band 3 that includes CH 38 and part of the CH-17 anion transport region.

Senescent cell IgG binds to band 3 denatured by NaDodSO₄, but does not bind to intact band 3 in situ in red cells (2,3). This suggests that a change in the tertiary structure of band 3 is required to initiate senescent cell IgG binding and removal of cells. Proteolysis of band 3 in situ could initiate such conformational changes.

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Figure Legends

Figure 1. Binding of antisera to band 3 and IgG eluted from senescent cells to band 3 and its proteolytic breakdown products. AB, Amido black; anti-band 3, antibodies to band 3; SC IgG, "senescent cell IgG" eluted from senescent red cells. Y, young cells; O3 old cells, fraction 3; O4, old cells, fraction 4. Cell populations were separated on Percoll gradients. Old cells are separated into four bands: old "fraction 4" being the densest, and old "fraction 3" the second densest. Old cells used for these studies represented 0.6% of the total cells. Cells were washed and membranes prepared. Polypeptides were transferred from 6-25% polyacrylamide gradient gels to nitrocellulose paper and incubated with antibodies to band 3 or IgG eluted from senescent RBC followed by ^{125}I labeled protein A. Polypeptides were stained with amido black.



Figure 2. ^{125}I labeling of the ~62,000 dalton proteolytic product of band 3 in intact cells. CB, Coomassie blue stain of RBC membrane proteins; AR, autoradiographs of membrane proteins from red cells labeled with ^{125}I Bolton-Hunter reagent. Lanes: A. RBC membranes; B. RBC membrane pellet after alkali treatment. Middle-aged red cells were washed and incubated with ^{125}I Bolton-Hunter reagent as described in the text. Membranes were prepared and analysed by NaDodSO₄/polyacrylamide gel electrophoresis. The gel was stained with Coomassie blue, dried, and exposed to film for six days.

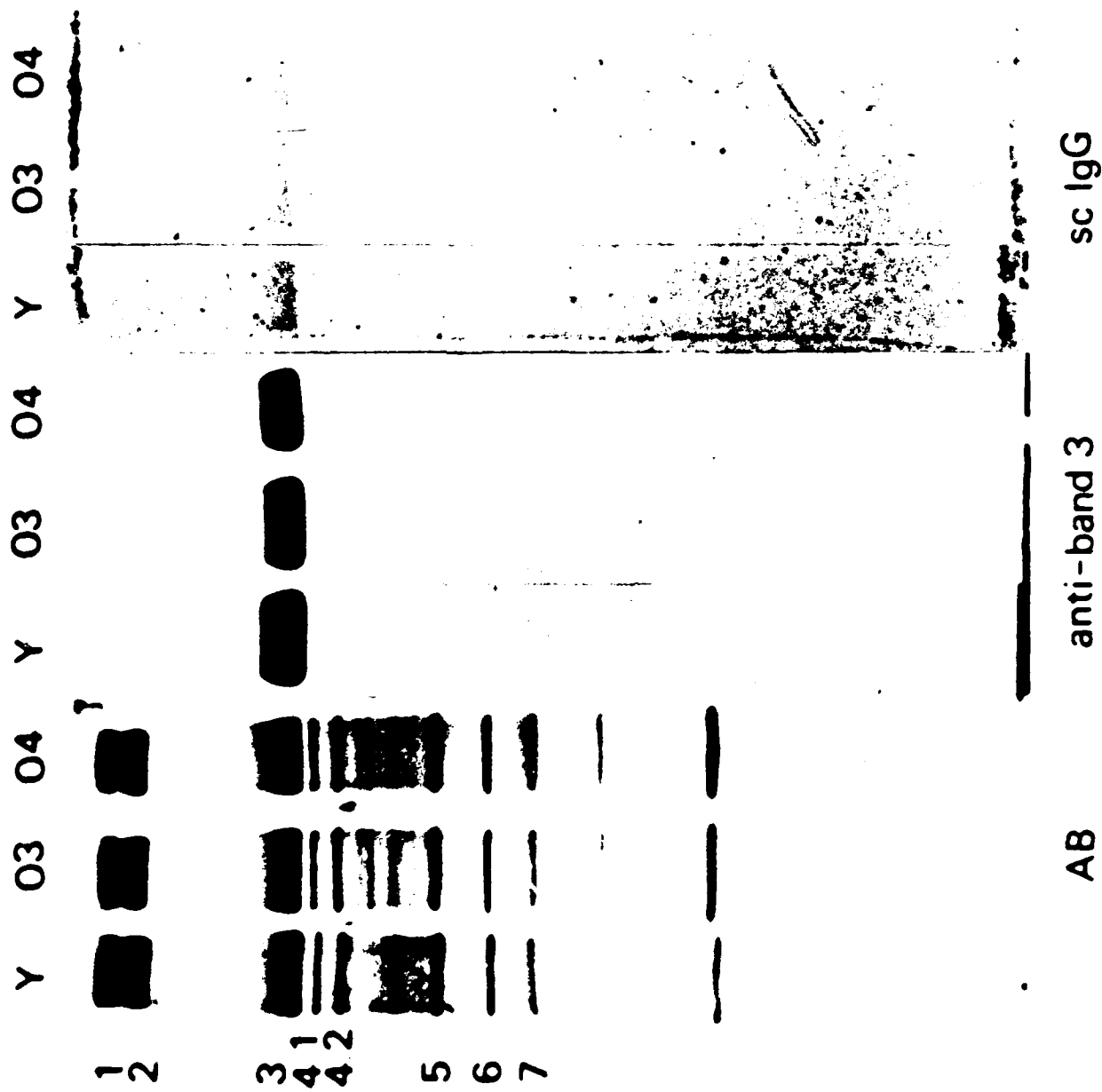


Figure 3. Binding of antiband 3 and IgG eluted from senescent red cells to band 3 and its proteolytic degradation products. Lanes: A, RBC membrane proteins without enzyme treatment; B, RBC membrane proteins from intact cells treated with α chymotrypsin overnight to generate CH-55 and CH-38; C, CH-17 following alkali treatment of membranes to remove peripneural membrane proteins; D, washed membrane pellet of alkali treated, inverted vesicles following removal of TR-41; E, supernatant from alkali treated, inverted vesicles containing TR-41. Red cell membrane proteins were separated on sodium dodecyl sulfate-polyacrylamide gradient gels (6-25%) and transferred to nitrocellulose paper. The paper was incubated with antiband 3 or IgG eluted from senescent red cells, washed, and incubated with ^{125}I labeled Protein A. The paper was dried and exposed to Kodak X-Omat RP film for five days at -80°C in a Cronex cassette with intensifying screens.

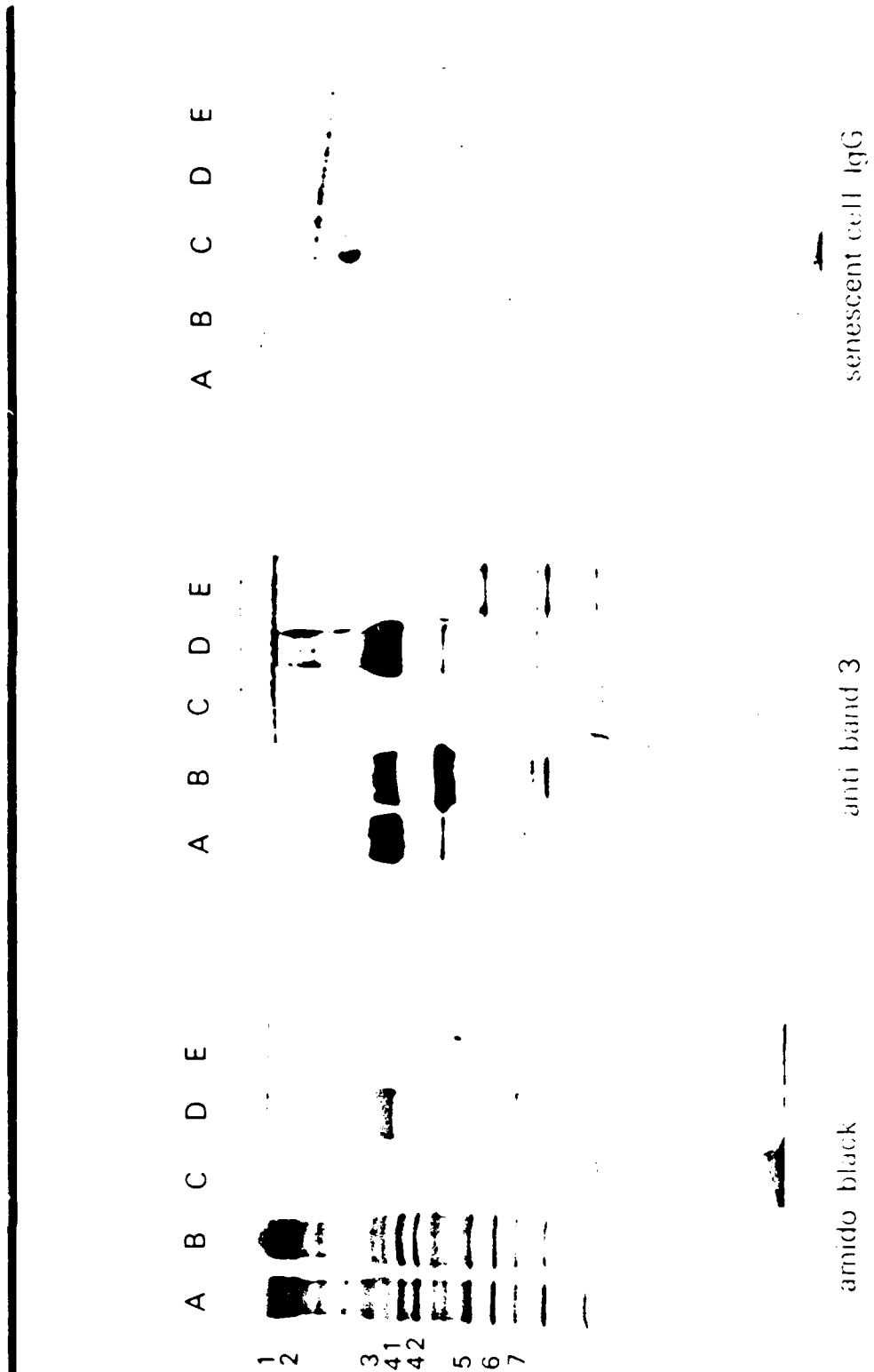


Figure 4. Two-dimensional peptide maps of band 3, its proteolytic fragments, and the senescent cell antigen. Panels: A, band 3; B, ~60,000 Da carboxyl terminal polypeptide complementary to TR-41; C. senescent cell antigen; D. CH-38; E. CH-17; F. CH-38 mixed with equal amounts of CH-17. O, origin; E, electrophoresis; TLC, thin layer chromatography.



Figure 5. Two-dimensional peptide map of CH-55.

A B A B

1
2
2.1

3
4.1
4.2

5
TD

CB

AR

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